workers in other scientific disciplines, in particular chemists, is now more strongly evident, largely because an array of scientific techniques is needed to characterize the microorganisms. The involvement of engineers is still lacking and consequently key questions are missed or omitted when considering the potential of microorganisms to treat complicated waste streams. Many engineers are surprised at the microbiologist's approach in tackling a seemingly new pollution problem. The technique of screening the polluted environment for thriving microorganisms is logical to the microbiologist, but curious to the engineer, who may not understand the subtleties of genera and strains.

It is this fusion of scientific and engineering approaches that is needed to enable bioremediation, and hence environmental biotechnology, to achieve its true potential.

Environmental legislation is now stringent and is likely to become even more so in the future. In this situation it is important not only that the process technology is understood, but also that the implications and consequences of perturbations to this technology can be accurately predicted. With environmental processes perturbations will undoubtedly arise, as to date there is no specification for effluents that is definitive.

Unfortunately, in the present commercial environment, the quest for scientific knowledge is too often perceived as no longer valuable or affordable. In this respect the success of biotechnology in other areas, e.g. pharamaceuticals, may well have a positive benefit to other markets, persuading nonscientists that knowledge and intellectual property is valuable and ignorance is unaffordable.

See also: **II/Ion Exchange:** Theory of Ion Exchange. **III/Biological Systems: Ion Exchange. Resins as Biosorbents: Ion Exchange.**

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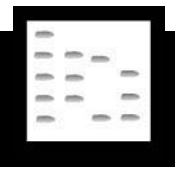
PrepBooks

METALLOPROTEINS: CHROMATOGRAPHY



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Classification and Characteristics of Metalloproteins

Metals are known to play essential roles in catalysis, macromolecular structure and membrane stabilization as well as hormonal and genetic regulation. Metals present at very low concentrations in tissues and biological fluids are termed oligoelements. Usually they do not occur in the biological matter as free ions, but as metal–protein complexes. The term metalloprotein is used to define a large group of proteins containing one or more atoms of metal bound to specific sites in the polypeptide chain. The binding sites on the protein are provided by histidine nitrogens, glutamate or aspartate oxygens and cysteine sulfurs; the metal ligand is usually represented by calcium, selenium, iron, zinc, copper and other heavy metals.

Metalloproteins can be divided into two groups: biologically active metalloproteins and proteins with

no apparent biological activity. The first ones include metalloenzymes and zinc-containing DNA-binding proteins. Metalloenzymes are particularly suited to studies on metal–protein interaction aimed at a better understanding of the enzymatic mechanisms; their activity is measured by means of specific assays with an appropriate substrate. The metal can either stabilize the protein structure or be part of the active catalytic site; sometimes distinct atoms of the same metal may have structural or catalytic roles, depending on the site to which they are bound.

Metalloproteins that do not exhibit biological activity can be isolated by simply monitoring the metal. The most widely used technique for metal determination is atomic absorption spectrometry (AAS); however, alternative methods are also available, such as flame atomic emission spectrometry, plasma emission spectrometry, differential pulse polarography and the neutron activation analysis.

Metalloenzymes

Metalloenzyme Preparation

Metalloenzymes are widely represented in almost every group of enzymes. The methods used for the purification and separation of these enzymes from other components do not differ substantially from those usually employed for enzymes not containing metals as prosthetic groups. These methods include techniques such as gel permeation, ion exchange, affinity chromatography and high performance liquid chromatography (HPLC). The reader is referred to these specific topics for details on the chromatographic techniques.

The purification strategy varies from one enzyme to another; however, an effective procedure for some enzymes is affinity chromatography. Alcohol dehydrogenase, for example, can be extensively purified by affinity chromatography based on the interaction of the coenzyme with a Blue A column and subsequent elution of the enzyme with NAD^+.

Good recovery of the metalloenzymes from chromatographic columns may depend on the precautions adopted during the separation procedures. In particular, special care is required to preserve the integrity of the metal–protein complex during chromatography by avoiding conditions that may affect metal binding. A loss of metal can occur in the presence of chelating agents such as ethylenediaminetetraacetic acid (EDTA) or as a result of decreasing the pH of the medium. Buffer complexation with metals may also affect metalloenzyme stability. The use of inorganic buffers should be avoided as they may remove metals essential to enzymatic activity. To avoid problems of metal chelation, the use of Good buffers (made of *N*-substituted taurine and glycine) is useful. Certain substances usually added to protein solvents may affect the metal binding; high concentrations of reducing agents such as dithiothreitol may remove metals with low binding affinity. In these cases, the best choice is to use 0.5% (v/v) mercaptoethanol as a reducing agent, as this substance does not remove metals.

Separation of Protein and Metal Moieties

Metal-free enzymes are well-suited to the study of the interaction of protein and metal ions and the effect of such interaction on the structure and function of the enzyme. Separation of metal from protein is easily performed using chelating agents at a pH between 5.5 and 7.5. The most commonly used chelator for apoenzyme preparation is 1,10-phenanthroline, but it is a good rule to test several chelating agents for their efficacy. A list of chelating agents used to remove the metal from various metalloenzymes is given in **Table 1**. The use of EDTA as chelator is not recommended because it binds to protein and laboratory glassware; if its usage cannot be avoided, it is advisable to add a trace amount of ^{14}C-EDTA to check its complete removal.

The procedure for apoenzyme preparation requires that the enzyme, at a concentration of 0.1–1 mmol L^{-1}, is dialysed against several volumes of chelator solution with several changes. Dialysis tubings may contain heavy metals, hence they should be heated at 70–80°C for 2 h in metal-free water (see below) before use. Metal-free dialysis membranes (Spectra/por) are also commercially available. The chelator is removed by extensive dialysis against metal-free buffer (see below). If 1,10-phenanthroline or 8-hydroxyquinoline is used to chelate the metal, their removal can be monitored by following the optical densities of the dialysate, because these

Table 1 Chelating agents commonly used for the preparation of metal-depleted enzymes

Substance	*Metal*	*Reference*
Ethylenediamine-tetraacetic acid	Zn^{2+} Mg^{2+}	McConn *et al.* (1964) *J. Biol Chel.* 239: 3706
1,10-Phenanthroline	Zn^{2+}	Prescott *et al.* (1983) *Biochem. Biophys. Res. Commun.* 114: 646
Dipicolinic acid	Zn^{2+}	Maret (1989) *Biochemistry* 28: 9944
8-Hydroxyquinoline-5-sulfonic acid	Zn^{2+}	Jacob *et al.* (1998) *Proc. Natl. Acad. Sci.* USA 95: 3489

substances absorb light. The chelator can also be removed by gel filtration chromatography. A suitable chromatographic system is represented by a Bio-Gel P polyacrylamide pre-loaded column (Bio-Rad). For most proteins, an exclusion limit of 6000 may allow recovery of the apoenzyme in the void volume with little dilution of the sample.

An alternative procedure for apoenzyme preparation is provided by the use of Chelex 100. The sodium form of the resin, previously equilibrated in metal-free buffer, is mixed with the metalloenzyme solution at a ratio of about 20% in volume. If the metal is essential for catalytic activity, a time-dependent loss of enzyme activity may be observed during the treatment with Chelex 100. The advantage of this method is that the apoenzyme so prepared can be stored in the presence of the resin, thus avoiding its reactivation by adventitious metals.

In order to maintain the protein in a metal-free form, it is important to minimize the presence of contaminating metals. One of the first precautions for preventing an unwanted reassociation with metals is the use of metal-free water in all purification and separation procedures. Ultra pure water suitable for apoenzyme preparation can be obtained by repeated distillation or by using a Milli-Q apparatus (Millipore). The latter system can supply water with a metal content below the detection limits of sophisticated analytical methods such as AAS, provided the cartridge is changed frequently.

Although metal-free water is indispensable, sometimes its use is not sufficient to eliminate contamination unless polystyrene metal-free containers are used for its storage. Of course, even the use of high purity water will not avoid unwanted problems if reagents, glassware and buffers themselves are sources of contamination. The use of polystyrene labware must be preferred in place of polyethylene and polypropylene, because these may be sources of metal ions. The ubiquitous presence of metals such as zinc, mercury, iron and aluminium in many laboratory reagents is another factor that has to be controlled. It is advisable to remove these metals from buffers and solutions before using them in separation procedures. The most widely recommended methods for eliminating metals from aqueous media are dithizone extraction or treatment with a chelating resin.

Dithizone (diphenylthiocarbazone) is used as a complexing reagent to remove heavy metals from aqueous solutions by exploiting its high solubility in organic solvents compared to the low solubility in water. Dithizone may be recrystallized by dissolving 2 g of substance in 100 mL chloroform. The volume of this solution is then reduced to one-half by evaporation under a nitrogen stream. The crystals are collected by filtration, washed with carbon tetrachloride and dried under vacuum. All the manipulations with organic solvents must be carried out in a hood. The solution to be extracted is shaken for 5 min with 0.1 vol of a freshly prepared dithizone solution (0.02% in chloroform) in a separatory funnel equipped with a Teflon stopcock (do not grease the stopcock). The extraction procedure must be repeated several times with different aliquots of dithizone solution. At the end, any trace of organic solvent present in the aqueous phase is removed under reduced pressure. This procedure works very well for ions such as Zn^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+} and Ni^{2+}, but is not as effective for Mn^{2+}. In addition, dithizone extraction cannot be used with buffers with a pH above 8, because at this pH dithizone solubility in water increases.

An alternative method uses a column of Chelex 100 as metal chelator. The resin is washed with 2 vol of 0.5 mol L^{-1} HCl, 5–6 vol of water and 2 vol of 0.5 mol L^{-1} NaOH. After a final wash with 5 vol of water, the resin is packed in a chromatographic column. The buffer to be demetallized is passed through the column at a flow rate of 10–20 mL min^{-1} cm^{-2}, and collected after several bed volumes have been discarded.

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Metalloproteins with no Enzymatic Activity

Detection of Metal-binding Proteins in Chromatographic Eluates

Most of the metalloproteins lacking catalytic activity are metal-binding proteins. In general, these molecules are characterized by a high metal-to-protein stoichiometry: usually from 4 to 7 atoms of heavy metal are bound to cysteinyl, glutamyl, aspartyl residues of the polypeptide chain. The best known among the metal-binding proteins are metallothioneins (MT), a family of cysteine-rich low molecular weight polypeptides present in all animal phyla, as well as in fungi, plants and cyanobacteria. Mammalian MT are single chain proteins made of 60 amino acids, including 20 cysteines arranged in two domains of metal–thiolate clusters containing 7 equivalents of metal (usually zinc, copper or cadmium).

Purification and separation of metal-binding proteins are performed by column chromatographic procedures, generally involving gel permeation and anion exchange methods and HPLC. As many metal-binding proteins do not exhibit evident biological activity, a widely used technique for monitoring chromatographic eluates is the determination of the metal by AAS. Flame AAS is mostly used for samples

in solution, provided the analyte concentration is in the order of p.p.m. (1 p.p.m. = 1 mg L^{-1}) and enough volume of sample (at least 1 mL) is available for analysis. If the analyte concentration is in the range of p.p.b. (1 p.p.b. = 1 μg L^{-1}), or if one wishes to minimize the volume of sample to be employed in the analysis, the technique of choice is the furnace AAS.

This technique, initially proposed by L'vov, became commercially available in 1969. A small amount of sample (20 μL) is introduced in a graphite furnace that is rapidly brought to high temperature by electrical heating. The sample is converted in atomic vapour and part of a light produced by a lamp containing the element to be analysed is absorbed by the analyte. In some cases, the sample is placed on a small platform added to the furnace to delay vaporization until the temperature within the furnace has reached a stable plateau. The addition of a matrix modifier may help to stabilize the analyte to high temperatures. A drastic reduction of the background influence is achieved by the Zeeman correction. The experimental conditions for the determination of the elements most commonly found in metalloproteins are reported in **Table 2** for graphite furnace AAS.

In general, the quantification of the MT-bound metal in chromatographic eluates does not require pretreatment of the sample such as digestion with oxidizing agents or ashing. However, the extent of matrix interference should be evaluated by measuring the absorbance of a known standard in the presence of the sample (internal standard technique). If metal concentration is high, interference may be reduced by appropriate dilution of samples. The determination of volatile elements such as cadmium and mercury requires the addition of a modifier (Table 2).

Separation of Metallothionein from High Molecular Weight Proteins

The procedure currently employed for MT isolation is gel permeation chromatography. In a typical preparation, 1 vol of an ethanol chloroform solution (1.05/0.08 v/v) is added dropwise to 1 vol of a tissue extract, previously centrifuged at 100 000 **g**. After the precipitate is centrifuged at 20 000 **g** for 15 min, the supernatant is mixed with 3 vol ethanol prechilled at $-20^{\circ}C$ and maintained at the same temperature overnight. The resulting pellet, collected by centrifugation at 20 000 **g** for 20 min, is dissolved in 20 mmol L^{-1} Tris/HCl buffer pH 8.6 and loaded on a Sephadex G-75 column equilibrated with 10 mmol L^{-1} Tris/HCl pH 8.6. The column is eluted with the equilibration buffer and the eluate is monitored for metal content. Such a procedure is particularly suitable for low molecular weight proteins like MT, because these can be easily separated from the bulk of high molecular weight proteins. MT usually elutes at the level of standard cytochrome *c* (mol wt about 12 000) because of molecular asymmetry (**Figure 1**).

One should be aware, however, that the original metal composition of MT may be altered by the presence of adventitious metals. As zinc usually found associated with tissue MT can be exchanged for other heavy metals with higher affinity for thiol groups, such as cadmium and copper ions, that may be present as contaminants in chromatographic media or glassware, it is advisable to render these sources metal-free before use. The methods for removing heavy metals from buffers have been described above. It is advisable to wash the gel permeation column with 3 bed vol of 2 mmol L^{-1} 1,10-phenanthroline to remove trace metals from the matrix. The column is then washed with metal-free buffer until disappearance of the absorbance at 320 nm.

Separation of Metallothionein Isoforms

Genetic polymorphism is a typical feature of metallothionein. One or more MT isoforms have been found in most animal tissues; their intracellular levels may vary from tissue to tissue. These isoforms are often very similar, with only a few amino acid substitutions in their amino acid sequences. Because they are so similar it is sometimes difficult to separate these proteins; however, if two isoforms have different

Table 2 Conditions for the determination of some elements by AAS with graphite furnace

Element	*Wavelength (nm)*	*Atomization temperature (°C)*	*Modifier*	*Graphite tube*
Cd	288.8	1600	0.2 mg $NH_4H_2PO_4$ + 0.01 mg $Mg(NO_3)_2$	Pyrolytic/platform
Cu	324.8	2300	None	Pyrolytic/platform
Co	242.5	2500	0.05 mg $Mg(NO_3)_2$	Pyrolytic/platform
V	318.5	2650	None	Pyrolytic/wall
Fe	248.3	2400	0.05 mg $Mg(NO_3)_2$	Pyrolytic/platform
Hg	253.7	2000	10 mg Te in 1% HCl	Uncoated/wall
Zn	213.9	1800	0.006 mg $Mg(NO_3)_2$	Pyrolytic/platform
Se	196.0	2100	0.05 mg Cu + 0.01 mg $Mg(NO_3)_2$	Pyrolytic/platform

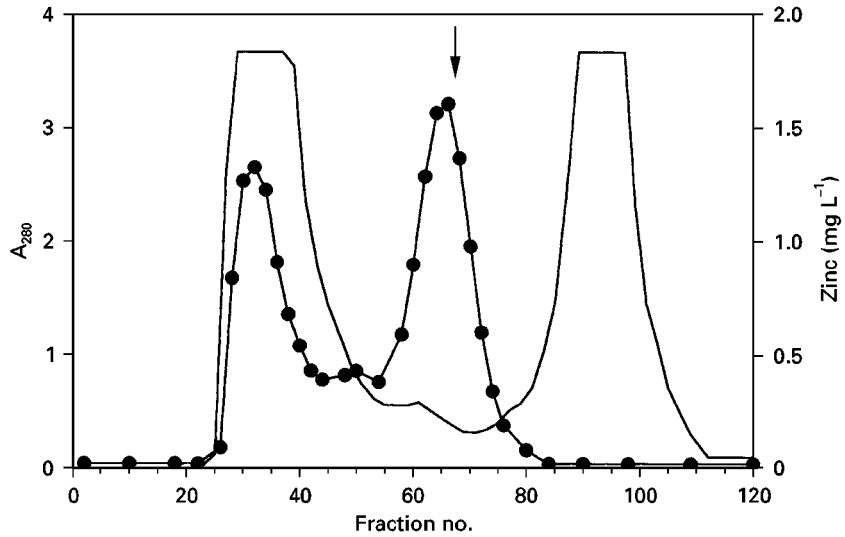

Figure 1 Separation of metallothionein by gel permeation chromatography. About 8 mL of extract containing 200 mg protein from fish liver was loaded on a Sephadex G-75 column (2.6 × 35 cm) previously equilibrated with 10 mmol L^{-1} Tris/HCl buffer pH 8.6. The column was eluted with the same buffer and fractions (2 mL) were monitored for absorbance at 280 nm (continuous line) and zinc content (circles). The arrow indicates the elution volume of standard rabbit liver metallothionein. Reproduced with permission from Scudiero R, Carginale V, Riggio M, Capasso C, Capasso A, Kille P, di Prisco G and Parisi E (1997) Difference in hepatic metallothionein content in Antarctic red-blooded and haemoglobinless fish: undetectable metallothionein levels in haemoglobinless fish is accompanied by accumulation of untranslated metallothionein mRNA. *Biochemical Journal* 322: 207–211.

net electric charges, they can be separated by anion-exchange chromatography. In **Figure 2** the elution profile from a diethylaminoethyl (DEAE)-cellulose column of two MT isoforms is shown. Metallothioneins were previously separated from high molecular weight proteins by solvent precipitation followed by gel permeation chromatography on a Sephadex G-75 column under conditions similar to those described above. Anion exchange chromatography was performed as usual, by loading the sample on the column; this was

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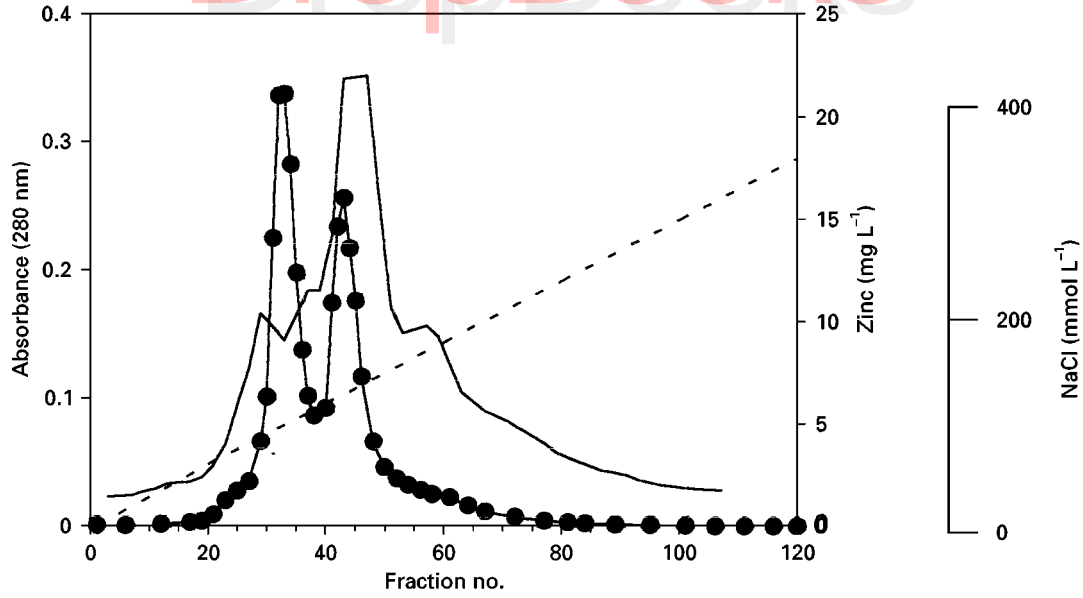

Figure 2 Separation of sea urchin metallothionein isoforms by anion exchange chromatography. The metallothionein-containing fractions from a Sephadex G-75 column were pooled and loaded on a DEAE-cellulose column (1.6 × 25 cm) equilibrated with 20 mmol L^{-1} Tris/HCl pH 8.6. The column was eluted with a linear gradient of NaCl (0–400 mmol L^{-1}) in equilibration buffer and fractions (3 mL) were monitored for absorbance at 280 nm (continuous line) and zinc content (circles). Reproduced with permission from Scudiero R, Capasso C, Carginale V, Riggio M, Capasso A, Ciaramella M, Filosa S and Parisi E (1997) PCR amplification and cloning of metallothionein cDNAs in temperate and Antarctic sea urchin characterized by a large difference in egg metallothionein content. *Cellular and Molecular Life Science* 53: 472–477.

Table 3 Chromatographic conditions for separation of metallothionein isoforms by HPLC

Column type	*Elution buffers*	*Running conditions*	*Reference*
TSK G 3000 SW (Yokyo Soda) (600 × 7.5 mm i.d.)	10 mmol L^{-1} Tris/HCl pH 8.6	20 min at a flow rate of 1.0 mL min^{-1}	Suzuki *et al.* (1984) *J. Chromatogr.* 303: 131
LiChrosorb RP-18 (10 μm) (Bischoff Analysentechnik) (250 × 21 mm i.d.)	A: 25 mmol L^{-1} Tris/HCl pH 7.5 B: 60% CH_3CN in buffer A	One-step linear gradient: 0–100 min, 0–40% B at a flow rate of 2 mL min^{-1}	Hunziker *et al.* (1985) *Biochem. J.* 231: 375
DEAE-5PW (Waters) (750 × 7.5 mm i.d.)	A: 10 mmol L^{-1} Tris/HCl pH 7.4 B: 200 mmol L^{-1} Tris/HCl pH 7.4	One step linear gradient: 0–12 min, 0–40% B at a flow rate of 1 mL min^{-1}	Lehman *et al.* (1986) *Anal. Biochem.* 153: 305
Aquapore RP 300 (Brownlee Laboratories) (10 μm) (250 × 4.6 mm i.d.)	A: 10 mmol L^{-1} Tris/HCl pH 7.5 B: 60% CH_3CN in buffer A	One-step linear gradient: 0–100 min, 0–40% B at a flow rate of 1 mL min^{-1}	Ebadi *et al.* (1989) *Neurochemical Res.* 14: 69
μBondapak C18 (Waters) (10 μm) (100 × 8 mm i.d.)	A: 10 mmol L^{-1} NaH_2PO_4 pH 7.0 B: 40% CH_3CN in buffer A	Two-step linear gradient: 0–5 min, 0–10% B; 5–20 min, 10–25% B	Richards (1989) *J. Chromatogr.* 482: 87

washed with the low ionic strength buffer and eluted with a linear gradient of NaCl.

A suitable procedure for separation of MT isoforms is HPLC, and this can be performed with various types of column systems. Conditions most usually applied for the HPLC separation of MT are given in **Table 3**. Since MT are converted in the corresponding metal-free apoforms at acidic pH, the use of neutral buffer systems is recommended. A suitable system is a μBondapak C_{18} column eluted with a two-step linear gradient of acetonitrile in phosphate buffer (**Figure 3**).

Preparation of Metal-free Metallothionein for Metal Reconstitution

Metal substitution in MT is a common procedure to produce a complex with a defined metal composition for structural studies. It must be remembered that certain metals form complexes with different coordination numbers: while Zn and Cd in MT are tetrahedrally coordinated, Cu forms tridentate complexes. To perform metal substitution it is necessary first to prepare the metal-free MT. In doing so, it is recommended that adventitious metals in reagents and glassware are avoided by following the procedures described above.

Metal-free MT can be prepared by lowering the pH of the protein solution to 2. Under these conditions, the metal moiety dissociates from the protein; the apothionein is then separated from the metal by gel permeation chromatography on a Sephadex G-25 column. An acidified sample of about 5 mL containing approximately 15 mg of protein can be separated

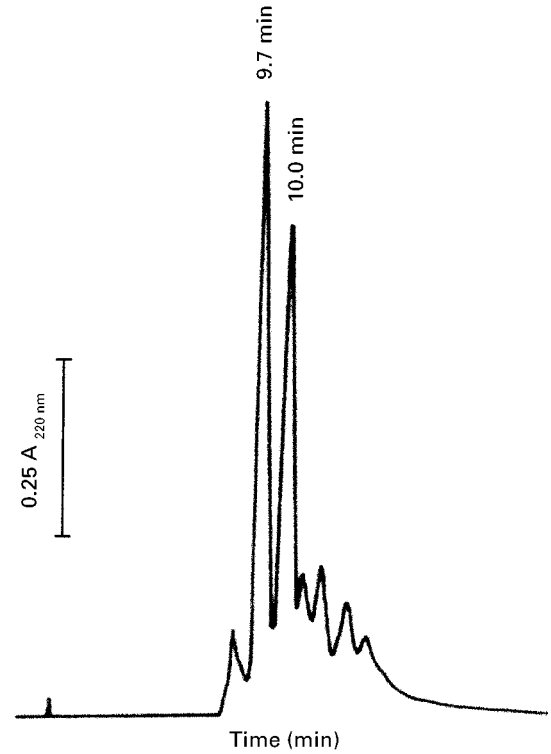

Figure 3 Separation of rabbit metallothionein isoforms by reversed-phase HPLC. The separation was performed on a μBondapak C_{18} column using a two-step linear gradient of acetonitrile in 10 mmol L^{-1} NaH_2PO_4 pH 7.0 at a flow rate of 3 mL min^{-1} (0–6% from 0 to 5 min, 6–15% from 5 to 20 min). The amount of purified MT used in each run ranged from 50 to 100 μg. Modified with permission from Richards MP (1991) Purification and quantification of metallothioneins by reverse-phase high-performance liquid chromatography. *Methods in Enzymology* 205: 217.

from the metal on a 2×20 cm column in 0.1% trifluoroacetic acid, by recovering the apothionein in the void volume. The addition of a reducing agent is not required as the sulfhydryl groups of cysteines are maintained in the reduced form as long as the pH is acidic. The apothionein so prepared can be stored at $-80^{\circ}C$ in an atmosphere of argon in a sealed ampoule. Prolonged storage should be avoided, as apothionein has a strong tendency to be oxidized. Anaerobic conditions are strictly required to prevent oxidation of the sulfhydryl groups with formation of large protein aggregates. The concentration of the demetallized protein can be determined by titrating the sulfhydryl groups with Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid)), or by measuring the absorbance at 220 nm and assuming an extinction coefficient of $7.9\ mg\ cm^{-1}\ mL^{-1}$ at pH 2. Metals such as zinc and cadmium are completely removed from the protein at low pH, but the procedure is not as effective for copper.

Removal of copper from Cu-thionein requires the use of chelating agents such as diethyldithiocarbamate (DTC). A solution of Cu-MT, brought to pH 5 by adding 30 µL of $3\ mol\ L^{-1}$ acetate buffer pH 5 per mL of solution, is mixed with solid DTC ($1\ mg\ mL^{-1}$ of MT solution) and incubated at room temperature for 1 h. The colloidal precipitate of Cu-DTC is then removed by filtration on a 0.22 µm Millipore filter, and the filtrate containing the apothionein is desalted on a Sephadex G-25 column equilibrated and eluted with 0.1% trifluoroacetic acid.

Metal substitution is performed by mixing an argon-purged acidic solution containing 20 mg apothionein with 7–8 equivalents of metal (Zn, Cd, Hg, Bi or Pb). The solution is then rapidly titrated to pH 8.6 (7.6 for Pb-MT) with an oxygen-free solution of $0.5\ mol\ L^{-1}$ Tris base under fast stirring. Immediately after titration, the solution is mixed with a small aliquot of a Chelex 100 suspension in $20\ mmol\ L^{-1}$ Tris/HCl pH 8.6 (7.6 for Pb-MT) and stirred for 5 min. The resin is then removed by centrifugation and the solution is concentrated by ultrafiltration. MT is purified by gel permeation chromatography on a Sephadex G-50 column equilibrated and eluted with $20\ mmol\ L^{-1}$ Tris/HCl pH 8.6.

Reconstitution with Cu(I) is more troublesome as this ion has a marked tendency to oxidation. A stable form of Cu(I) can be prepared by dissolving Cu_2O in acetonitrile containing $2\ mol\ L^{-1}$ $HClO_4$ at $100^{\circ}C$. The solution is evaporated at room temperature to obtain crystals. Reconstitution is carried out by mixing apometallothionein with increasing amounts of a solution of Cu(I)-acetonitrile at pH 2 followed by titration to pH 7 with Tris/acetate buffer. The whole procedure must be carried out under anaerobic conditions.

Conclusions

Developments in the field of metalloprotein research are strictly related to any future progress achieved in protein separation techniques. For metalloproteins, the main goals are reduction in the amount of the biomass required for their preparation, and decrease in separation time. Large amounts of protein are expected to be produced by expanding the use of recombinant DNA techniques combined with effective separation procedures such as affinity chromatography. Immunochemical methods can be applied for the detection of small protein quantities present in chromatographic eluates, and can be particularly useful for proteins lacking enzymatic activity. Techniques coupling HPLC and AAS may contribute to decreasing the time gap between sample separation and metal detection.

See also: **II/Centrifugation:** Analytical Centrifugation. **Chromatography: Liquid:** Mechanism: Size Exclusion Chromatography. **III/Peptides and Proteins:** Liquid Chromatography. **IV/Essential Guides for Isolation/Purification of Enzymes and Proteins.**

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